HeMicro Sequential Injection Lab-On-Valve for Process Monitoring and Bioanalytical Assays

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Abstract

This article illustrates the basic principle and operation of micro sequential injection Lab-On-Valve system (μSI-LOV) and the applications of the μSI-LOV system as a Process Analytical Technology (PAT) system for process monitoring and bioanalytical testings. The μSI-LOV system has also been used as an interface to acquire process samples for other auxiliary analytical instruments such as HPLC, CE, and MS. As an interface, the μSI-LOV enables traditional analytical instruments having process monitoring capability, and becomes part of the PAT system.

One of the applications listed in this article, real-time on-line mAb titer monitoring using affinity chromatography enabled μSI-LOV system, is highlighted and illustrated. The titer monitoring was demonstrated over a research scale without human intervention throughout the course of the fermentation process. Two protein A columns were attached to the LOV manifold, for monitoring up to two cell cultures. The chromatography bind and elute conditions were adopted from the off-line HPLC method. The titer results derived from the on-line monitoring were compared to the off-line analysis, and the result with good correlation was achieved.

Introduction

One of the key objectives for an automated analytical system is to fulfill the needs of the laboratories that require high-throughput routine analysis of a wide spectrum of species. The automated analytical system should consist of precise sampling and control of analytical events that can ensure the assay conditions are well controlled. High accuracy and reproducibility over an extended period of time should be achieved compared to other off-line analysis instruments. The flow injection analysis system (FIA), sequential injection analysis system (SIA), and the most recent μSI-LOV instrumentations have been proven to meet the requirements of laboratory automation with inherent features of high efficiency, high precision, and a broad spectrum of versatility.

Process analytical technology (PAT) is a new FDA initiative for at-line, in-line, and on-line quality control of pharmaceutical production. A PAT system has been defined by the FDA as:

"...a system for designing, analyzing, and controlling manufacturing processes through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality."

The PAT initiative focuses on building quality into the product and manufacturing processes, as well as continuous process improvement. The μSI-LOV system has been evaluated and preliminarily identified for its potential to be part of the PAT-enabled system to fulfill the objectives of PAT and is suitable for online monitoring and process control applications.

Evolution of SIA from FIA

Developed by Ruzicka and Hansen [1,2], the flow injection analysis (FIA) system performs assay in a format in which the continuously flowing carrier stream pushes the sample and reagent forward at the upstream of the system. The reaction product is detected by a detector located at the downstream of the system. It is an automated technique that allows for easy optimization and for routine laboratory analysis. A typical FIA system (Figure 1) consists of a peristaltic pump to drive the carrier, a sample injector with bypass channel allowing continuous flow of the carrier solution, a mixing coil, and a detector. The sample injector in a conventional FIA system employs a two position valve with a sample loop with a fixed volume, and a peristaltic pump can only provide a fixed carrier flow rate. Therefore, the injected sample volume and reaction time can not be easily adjusted by a computer or optimized by software without physically reconfiguring the system. The order of the assay events in the FIA system (such as dilution, mixing, separation, etc.) is fixed and strictly bonded to the system hardware configuration.

The sequential injection analysis (SIA) system, the second generation FIA system, was introduced by Ruzicka and Marshall [3] with the aims of improving flexibility, reducing sample and reagent consumptions, and allowing the technique to be more compatible with computer automation. A typical SIA system (Figure 2) comprises a bi-directional syringe pump, a holding coil, and a multi-position valve

(MPV). A detector is one of many peripherals of the SIA system and can be installed to the desired port on the MPV. The syringe pump and the MPV are controlled by a computer with dedicated software. While a conventional FI protocol needs physical reconfiguration of the flow manifold to perform different assays, the computer controlled SIA system allows all experimental protocols to be randomly executed. In a typical assay cycle of the SIA system, the sample and the reagent are injected sequentially through the MPV into the holding coil, forming a well-defined solution profile. This profile is then transported through the mixing coil and into the detector by the flow reversal action provided by the syringe pump after the MPV has been switched to the detector port. Flow programming, along with random access to sample and reagent ports, provides outstanding versatility since all operating protocols such as sample injection, mixing, and separation are controlled precisely by the software without need for physical reconfiguration of the system.

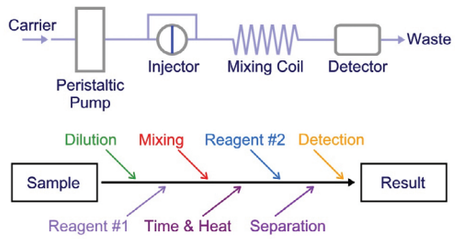

Figure 1. Schematic diagram of a FIA system and its operations

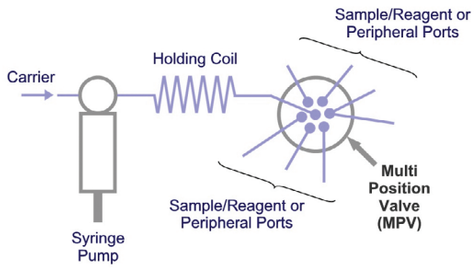

Figure 2. Schematic diagram of a SIA system to its simplest form

SIA System Integrated with Lab-on-Valve Manifold

The combination of SIA system and the LOV manifold becomes the µSI-LOV system (Figure 3a). Use of the LOV manifold allows further downscaling of a conventional SIA system by integrating sample, reagent, and analytical peripherals within a micro machined structure (Figure 3b). The central port of the LOV manifold is usually connected to the holding coil of the SIA system, allowing the central port random access to the six satellite ports through a shallow channel grooved on the rotating surface of the MPV. Large channel diameter (1.61 mm) allows the LOV manifold to accommodate particulate substances and prevent clogging if un-treated real-life samples were used. The design of using a smooth inner surface and short channel distance is intended to minimize carry-over between assays. Unique fluid channel geometry promotes convective mixing in a short distance to induce maximum production of reaction products in case reagent chemistries. The LOV manifold is compatible with detection systems such as UV-Vis absorbance, fluorescence, NIR, etc. It can also serve as an interface to auxiliary analytical instruments such as electrospray mass spectrometry or capillary electrophoresis by assigning specific ports to be connected to the auxiliary instruments (Table 1).

Operation of the µSI-LOV System

Successful operation of a flow-based system requires precise mixing, delivery of sample and reagent, and well-controlled experimental factors (e.g. temperature, flow rate, and incubation period). The computer-controlled µSI-LOV system allows these conditions to be further maintained over an extended period of operation time. With strict control of assay throughout the entire analysis period, what happens to one injected sample is exactly the same for any others. Reproducible timing in the µSI-LOV system is achieved through the repeatability of all events in a complete sample measurement cycle, including sequencing of sample and reagent zones into the holding coil, transportation of stacked zones into the detector, and the duration of the data acquisition period. There are two driving forces that cause the injected zones to mix and to spread into each other: (1) an internal one which is symmetrical and is due to intrinsic molecular diffusion, and (2) an external one which is due to the bulk carrier medium that transports the whole sample zone through the system, or the dispersion. The formation of the reaction products and the degree of completion of the reaction result from the combined effects of diffusive mixing and dispersive mixing.

Zone Gradient Overlapping in the µSI-LOV

Another important feature of the µSI-LOV system is that the product from the sample and reagent reaction can be consistently obtained due to the fact that the system can generate reproducible dispersion in the stacked sample and reagent zones. Through software programming, one can manipulate the amount of sample and reagent in the µSI-LOV system, thus allowing the selection of a certain concentration gradient in the dispersed sample solution profile to precede the chemical reactions.

A composite solution zone in a µSI-LOV system contains impulse components of sample (S) and reagent (R). The concentration gradients of S, R, and the product (P) will form as a result of the mixing of components S and R (Figure 4a). The formation of the concentration gradients resulting from the penetration of each component is due to the flow rate at the central streamline. It is twice as much as the mean flow velocity for the total fluid. The area of this mixing will increase with time and travel distance.

Since the reaction product is formed at the contacting parabolic surface of sample and reagent zones, it is important to maximize zone overlapping (or mixing) in order to gain the maximum product yield. This can be accomplished by increasing the magnitude of the flow rate under fixed channel geometry. As the stacked sample and reagent zones are aspirated into the holding coil (HC), radial dispersion is promoted due to the introduction of the secondary flow, by sudden geometry change (some 90° turns) of the fluid channel in the LOV manifold (Figure 3b). The increased dispersion promotes the mixing of the sample and the reagent, and increases the product yield. During the flow reversal, the inversed flow direction first promotes the radial mixing through local turbulence, and then further axial dispersion and zone overlapping. Upon arrival of the stacked zones to the detector, more

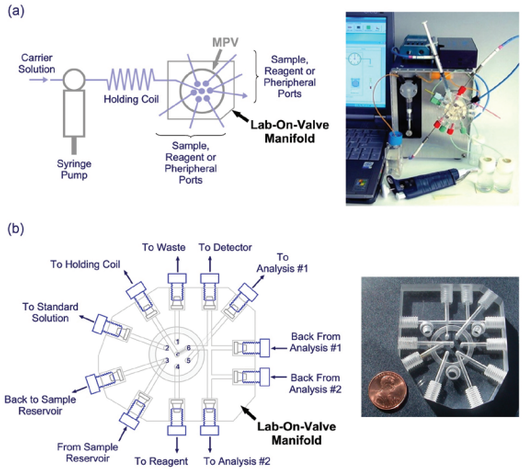

Figure 3. The µSI-LOV system and the Lab-On-Valve (LOV) manifold

(a) Schematic diagram of the µSI-LOV system (left) and its picture (right). A complete µSI-LOV system consists of a syringe pump, a holding coil, a multi-position valve (MPV), the LOV manifold, and sample detection peripherals (optical fibers, light source, and detectors). On the top of the MPV is the LOV manifold, which serves as sample processing hub of the µSI-LOV system, as well as the interface to auxiliary analytical instruments. Appearance of the µSI-LOV system is quite compact overall.

(b) Magnified diagram (left) and picture (right) of the LOV manifold. The LOV manifold has six reagent ports (1-6) and a central port (c) that is connected to the holding coil. It is made of transparent PMMA (polymethyl-methacrylate) and could also be made of polyetheretherketone. Port assignments shown are for demonstration purpose only. Actual port assignment should base on specific assay needs.

intense secondary flow is provided by the unique channel geometry of the LOV manifold to ensure more complete mixing of the sample and the reagent. The reaction product is then measured in the detector. In summary, the introductions of the zone overlapping and the secondary flow, in which magnitudes are adjustable by flow reversal and flow acceleration, are the key mixing strategies used in the µSI-LOV system.

Product Detection Modes in µSI-LOV

Apart from conventional batch-mode operation, a chemical reaction implemented in the µSI-LOV system does not need to reach equilibrium (i.e., reaction reaches end-point). Since all chemical reactions are time dependent, reproducible timing of sample handling operations is critical to the success of flow-based chemical assays.

Continuous-flow mode

Product delivery in the continuous-flow mode means that the carrier stream is steadily flowed throughout the system and the injected sample is delivered by the carrier stream at the same flow rate to the detector (Figure 4b). The time interval available for the chemical reaction is defined by the linear flow rate of the carrier stream and the distance between the point of the injection and the detector. Under a fixed carrier flow rate, a longer channel provides longer reaction time; however, the reaction product yield is offset by dispersive dilution due to increase in axial dispersion of the product zone. With flow conduits unchanged, elevated flow rate can promote mixing and boost sample throughput but will increase waste production and lead to insufficient production of the reaction product. Conversely, slower flow rate allows longer reaction time and higher product yield but will sacrifice sample throughout and increase the possibility of sample carry-over effect.

Stopped-flow mode

The stopped-flow mode is based on delivering a selected position of the stacked sample and reagent zones to the detector (Figure 4c). When the stacked zones are on their way to the detector, the reaction has not reached equilibrium. Upon arrival of the desired position on the stacked zones at the detector, the carrier flow stops and the reaction rate curve is then recorded while the reaction product is being formed at the detector. Optimization of reaction conditions can be automated through the selection of sample-to-reagent volumetric ratio by adjusting the flow reversal volume so that the position of optimized sample-to-reagent volumetric ratio can be located. After acquiring the data, carrier flow is resumed and the remaining sample and reagent zones are flushed out of the detector; the baseline is then restored. Data acquisition in this operating mode provides information on reaction kinetics through reaction rate measurement.

The stopped-flow mode provides longer reaction time for higher sensitivity. Sensitivity is promoted by adjusting the duration of the stopped flow period, which allows optimization of the signal-to-baseline ratio, as slower chemical reactions can be given a longer time to proceed. It also allows miniaturization by shortening the length between the injector and detector so that the dispersive dilution of sample and reagent is reduced. It thus saves reagents and generates less waste than the continuous-flow mode since the µSI-LOV system operates the syringe pump intermittently rather than continuously.

µSI-LOV System for Process Monitoring

The µSI -LOV system has been used to monitor mammalian and *E. Coli.* cell cultures (Table 1). Product titer is one of the most important performance attributes of the bioprocess. Automatic and sterile sampling and sample processing have been developed for off-line and on-line monitoring of reactors.

On-line, real-time mammalian cell culture mAb titer monitoring was demonstrated on a µSI-LOV incorporated with two protein A (ProA) columns. The entire monitoring task was accomplished without human intervention throughout the course of the cell culture process. Two ProA columns were attached to the LOV manifold: one column was used for on-line titer monitoring, while another column was reserved for a second bioreactor of a different mAb cell culture to prevent cross-contamination (Figure 5). Binding and elution buffers were ordinary buffers used in affinity chromatography. The mAb titer detection mode was continuous-flow mode at 280 nm. Calibration curve with the titer dynamic range of 500 to 4500 mg/L ($r2 = 0.9997$) was automatically generated at the beginning and the end of the on-line monitoring (Figure 6a). The amount of sample needed through a ceramic membrane filter per assay was 60 µL (RSD% for each sample at each time point < 2.0%, triplicate injections). Assay control samples were injected after each real-time sample to ensure suitability of the

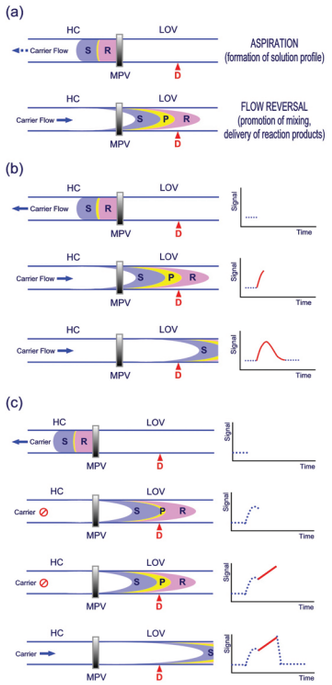

Figure 4. Zone dispersion and product detection modes (a) Formation of a solution profile in a micro sequential injection system. A simplified µSI-LOV system consists of a syringe pump (not shown in the figure) located at the upper stream of a holding coil (HC), a MPV provides random access of sample (S) and reagent (R) through the LOV manifold, and a detector (D) is located at the downstream of the system. After the aspiration of the solution profile containing sample and reagent zones into the HC (above figure), the degree of sample and reagent mixing is then promoted by the flow reversal action of the system (below figure) and the reaction product (P) is then delivered and detected.
(b) Continuous-flow mode. Sample and reagent are aspirated sequentially into the HC, forming a solution profile. The solution profile is delivered to the detector via the flow-reversal action provided by the syringe pump. The product signal is then collected (red peak). Since the carrier is flowed continuously, the product response is collected in the form of a peak.
(c) Stopped-flow mode. Sample and reagent are aspirated sequentially into the HC, forming a solution profile. After delivering the solution profile into the detector, carrier flow stops, and the reaction product begins to form. During the stopped-flow period, the increase in the formation of P contributes to a rising product signal at the detector (red straight line). The slope (or the height at the highest point of the line) represents the sample response. Upon the completion of the measurement, the detector is flushed and the signal returns to baseline.

assay system (RSD% = 1.7% at 1500 mg/L, n = 10). The titer results derived from the on-line monitoring were compared to the off-line conventional ProA HPLC results, and good correlation was achieved (Figure 6b). The point of challenge of the whole monitoring system is the sampling interface. Sampling probe fouling due to filtration membrane clogging and minor mAb adsorption, especially at the late stage of the cell culture, was noted.

By closely monitoring the titer during the cell culture process, some reflection points were observed indicating unique activities during the cell culture (Figure 6c). The information could potentially lead to further optimization of culture conditions and feed strategies. Information like such could easily be overlooked if only off-line sample were analyzed.

System Configurations	System Design	Applications	Reference
µSI-LOV-ProA Columns	On-line sterile sampling and analysis	On-line mAb titer monitoring	Manuscript in preparation
µSI-LOV-CEX Column	In-line sample preparation and analysis	Direct amino acid analysis of cell culture media	Manuscript in preparation
µSI-LOV-RP-AEX Columns	In-line sample matrix removal, preconcentration, and analysis	Direct product quality assessment for cell culture media	Manuscript in preparation
µSI-Bead Injection-LOV	In-line sample matrix removal, preconcentration, and analysis	Affinity assays of immuno globulin on protein immobilized beads	[4]*
µSI-LOV	On-line sterile sampling and analysis	Fermentation monitoring of cell culture substrates	[5]
µSI-LOV-Micro Column	In-line sample pretreatment and analysis	Environmental monitoring of pollutants	[6]
µSI-LOV-Micro Column-ICP-MS	In-line sample enrichment and analysis	Determination of metals in human urine samples	[7]
FI/SI-Micro Column-ETAAS	In-line sample enrichment and analysis	Determination of metals in environmental and biological samples	[8]
µSI-LOV-CE	In-line sample dilution, delivery, and analysis	Anion separation and indirect detection by CE	[9]
µSI-LOV-CE	In-line sample derivatization, delivery, and analysis	Automatic protein derivatization and CE fluorescence detection	[10]

* Sources listed in References

Table 1. Selected applications using µSI-LOV system

µSI-LOV System for Other Bioanalytical Assays

Versatility of the µSI -LOV system has been demonstrated in a number of selected applications listed in Table 1. In some cases the LOV was used as a sample processing *front-end* to auxiliary analytical instruments such as UV-VIS spectrometry and electro thermal atomic absorption spectrometry (ETAAS), as well as mass spectrometry (MS) and capillary electrophoresis (CE) for environmental, biotechnological, clinical, and trace element assays.

Conclusion

In conclusion, the µSI-LOV system has the following features:

- Versatility: The µSI-LOV system contains open architecture components, which allow additional peripherals to be added to the core instrument, and enable in-line analysis for process control offering an instrumentation aimed for special assay needs.
- Economics: The system provides low sample and reagent consumptions by bringing sample and reagent into an integrated LOV manifold where distorted channels geometry promotes convective mixing effectively in a short distance in close vicinity.
- Robustness: The system is able to accommodate to particulate substances and prevent clogging due to relative large channel diameter in the LOV manifold. Smooth inner surface to minimize carry-over between assays.

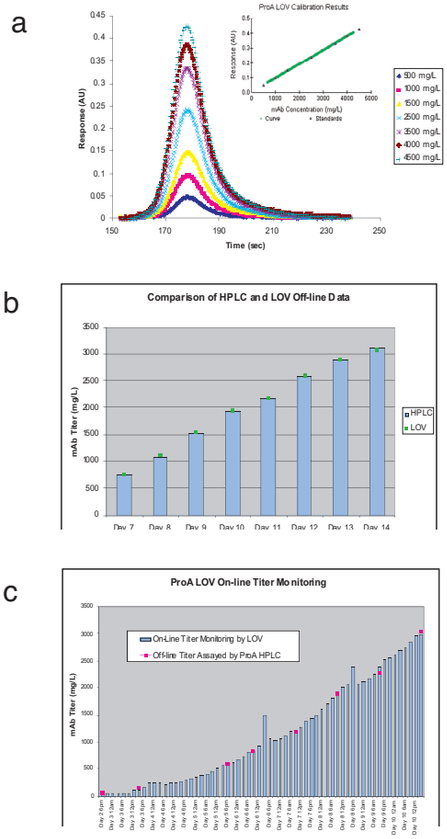

Figure 6. On-line monoclonal antibody titer monitoring
(a) Calibration of mAb on an affinity column using the µSI -LOV system (r2 = 0.9997).
(b) Comparison of conventional protein A HPLC results and off-line batch results from µSI -LOV system coupled with an affinity column (green dots, RSD% for each sample < 2%, n = 3).
(c) On-line titer monitoring results performed by µSI -LOV system compared to off-line protein A HPLC results (red dots). Sampling frequency for on-line monitoring: sample every three hours.

- Computer-operation: Flexible experimental protocol through software allows precise timing control of assay events and integrated peripherals while collecting and evaluating data and provides feedback mechanism through software control governed by preset assay thresholds.
- PAT-enabling system: Hyphenated system design enables conventional analytical instruments having process monitoring and control capabilities.

Successful implementation of the µSI-LOV system will be beneficial to the process development and manufacturing sites by:

- Offering labor-free sampling and assay routines from process stream. Computer controlled routines ensure frequent sampling as desired and maintain process stream sterility, as well as improves operator safety and reduces human errors.
- Reducing sample turn-around time by providing instant assay results on-site for continuous strategic process optimization, which leads to reduction in optimization and production cycle.
- Verifying non-conformance at early stage of the process to reduce cost.
- Performing automatic data capture and providing real-time automatic control and continuous feedback on manufacturing process that will ensure peak process performance and maintain product quality at all time.
- Enabling PAT capability to other analytical instruments by coupling the µSI-LOV system to other analytical instruments.

In recent years, analytical spectroscopic instruments such as Raman and NIR have been refined and miniaturized to incorporate optical fiber technique that enable them to be compatible to the µSI-LOV system. Anticipated advantages of applying the µSI-LOV system to process stream are not only automation of process monitoring and control, but also consolidation of process understanding that lead to consistent product yield with the acceptable product quality and reduction in operating cost.

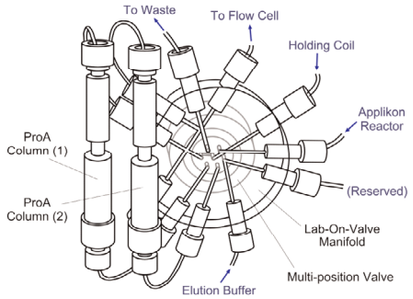

Figure 5. The configuration and port assignment of LOV manifold for on-line mAb titer monitoring. The binding buffer was used as the carrier buffer throughout the system. The ProA column (1) was used for the on-line titer monitoring demonstrated in this article. The ProA column (2) was installed and could be used for the monitoring of a second cell culture process.

References

1 Ruzicka, J. and Hansen, E. H., Anal. Chim. Acta, 1975, 78, 145.
2 Ruzicka, J. and Hansen, E.H, "Flow Injection Analysis" 2nd ed. J. Willey, N.Y. 1988.
3 Ruzicka, J. and Marshall, G. D., Anal. Chim. Acta, 1990, 237, 329.
4 Jaromir Ruzicka., Analyst, 2000, 125, 1053-1060.
5 Chao-Hsiang Wu, Louis Scampavia, Jaromir Ruzicka, Bruce Zamost, Analyst, 2001, 126(3), 291-297.
6 Chao-Hsiang Wu, Jaromir Ruzicka, Analyst, 2001, 126(11), 1947-1952.
7 Jianhua Wang and Elo Harald Hansen, J. Anal. At. Spectrom., 2001, 16(12), 1349.

8 Jianhua Wang and Elo Harald Hansen, Anal. Chim. Acta, 2000, 424, 223.
9 Chao-Hsiang Wu, Louis Scampavia, Jaromir Ruzicka, Analyst, 2002, 127(7), 898-905.
10 Chao-Hsiang Wu, Louis Scampavia, Jaromir Ruzicka, Analyst, 2003, 128(9), 1123-1130.

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